2. The Leaf Surface as a Substrate for Colonization by Fungi

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INTRODUCTION

The colonization of leaf surfaces by fungi presents an interesting study in substrate relationships. The living leaf can act as a landing stage for spores and other propagules in the air, whether they are deposited by gravity, boundary layer exchange, impaction or water splash. Once on the leaf, unless they are washed off by rain, they derive benefit by diffusion of nutrients from the leaf, and from algae and pollen grains which are present on the leaf surface. When leaves are infested with aphids, the honey dew forms an abundant source of food. Although the earliest fungal arrivals on the leaf surface can grow and develop without hinderance from competitors, the picture is not all to the advantage of the potential colonizer. While transpiration may mitigate against extreme levels of relative humidity, the leaf surface is not insulated against temperature fluctuations. The fungi on the leaf surface are also exposed to the ultra-violet radiations in daylight.

The earliest colonizing fungi are thus aided by an absence of competition and an actual, or at least a potential supply of nutrients, but they have to germinate and grow in a relatively hostile environment.

The presence of fungi on the surface of plants has been recognized for more than a century; in 1866 de Bary described *Dematium pullulans* as a fungus commonly occurring in this situation. The early description of the sooty moulds (the "soot-dew" of Zopf) and the brown-walled cells found on succulent fruits by Pasteur were referred to later by de Bary (1887). During the last 20 years, interest has become increasingly centred on the leaf surface as a habitat for fungi. It is here that the parasitic fungi must spend a critical time until they can establish an infection; similarly, saprophytes and weak parasites which begin the decomposition of the tissues must wait until a lowering of the host's resistance, or the onset of senescence allows the initial penetration into the tissues.

The present study has been carried out on leaves of sycamore (*Acer pseudoplatanus*). This plant was selected for studying the early stages of leaf colonization because the leaves are rapidly decomposed after abscission. It was hoped to see whether the initial colonization occurs earlier than on other leaves, and to see if the process of colonization is telescoped in any way.

MATERIALS AND METHODS

Leaves were collected at monthly intervals from the first opening of the buds throughout the life of the leaf, until after leaf fall and complete disintegration of the leaves. The collection site was near Winkburn, Nottinghamshire (Grid Reference 725 593) where there is a pure stand of sycamore.

After collection, the leaves were transported to the laboratory in new polythene bags, and were then treated in one of the following ways:

1. Spore fall method

Whole leaves were cut in half longitudinally. Each half leaf was attached to the lid of a 13 cm petri dish by either its upper or its lower surface so that the fungi on both surfaces were free to fall on to the potato dextrose agar (P.D.A.) for 24 h. The plates were then scanned for fungal growth, and new lids, without leaves, were fitted.

2. Leaf clearing

3 mm squares, cut at random from the leaves, were immersed in chloralhydrate at a concentration of 5 g per 2 ml of water until the tissues had cleared (Shipton and Brown, 1962). They were then scanned microscopically, normally after staining with lactophenol-cotton-blue. During these observations, the relationship between spores and leaf anatomy was noted.

3. Scanning Electron Microscopy

7 mm discs, cut at random from the leaves, were mounted on solid metal specimen stubs using silver dag as the adhesive. They were then coated under vacuum with gold to give a 150 Å layer, and were placed in a desiccator before being examined in the "Stereoscan" Electron Microscope (Cambridge Instrument Co.).

4. Cultural methods

(*a*) *Washed leaves*. 3 mm leaf squares were cut at random and were serially washed (Harley and Waid, 1955) for 2 min in each of 30 changes of sterile water. After blotting dry on sterile filter paper they were plated out on P.D.A. and on cellulose agar (C.A.), and then incubated at 25°C. (*b*) *Surface-sterilized leaves*. 3 mm leaf squares were cut at random and were immersed in mercuric chloride (0·1% w/v) for one minute. They were then serially washed in 5 changes of sterile water, plated out on P.D.A., and incubated at 25°C.

5. Leaf digestion

3 mm leaf squares which had been surface-sterilized with mercuric chloride were digested using pectinase ("Macerozyme": Kinki Yakult Mfg. Co., Nishinomiya, Japan) until only the veins remained intact. These were washed in sterile water before plating out on P.D.A.

6. Exposure to ultra-violet radiations

Spore suspensions of the common leaf surface fungi and other species were exposed to ultra-violet light, using a Hanovia bactericidal lamp (wavelength 2537 Å, plates exposed 50 cm from the tube). The plates were removed after various intervals, up to a maximum exposure time of 35 min.

RESULTS

1. Spore fall method

The distribution of fungi revealed by this method showed that *Sporobolomyces* was present predominantly on the upper surface of the leaves, where it was found mainly along the veins. The seasonal increase of this fungus on the two leaf surfaces is illustrated in Fig. 1. On the lower surface, *Cladosporium herbarum* was the most frequently isolated species, and *Sporobolomyces* was infrequent compared with its abundance on the upper surface. *Aureobasidium pullulans* was isolated infrequently from both surfaces.

2. Leaf clearing

When examining the surfaces of green leaves which had been cleared immediately after collection, the most striking feature was the presence of fungi along the veins, frequently with the fungal cells orientated parallel with the vein axis (Fig. 2 A and B). In the case of both *Aureobasidium* and *Sporobolomyces*, the fungal cells lay over the epidermal cell walls which were perpendicular to the leaf surface, whereas those of *Cladosporium* were randomly distributed over the veins. The veinal distribution of *Aureobasidium* was plotted, using an eye-piece grid, and the results showed that this species was significantly associated with the veins (P = 0·001).

On the leaf surface, *Aureobasidium* occurred as individual cells for only a very limited period of time. Within three weeks of the bursting of the buds, the form of this fungus had changed, and it was present as clusters of thick walled cells which appeared to be embedded in mucilage (Fig. 3 A and B). From July onwards, *Aureobasidium* was present in the form of "fumagoid structures". These were dark, thick-walled chlamydospores which were sometimes linearly arranged, and, infrequently were clustered to give the appearance of microsclerotia (Fig. 3 C).

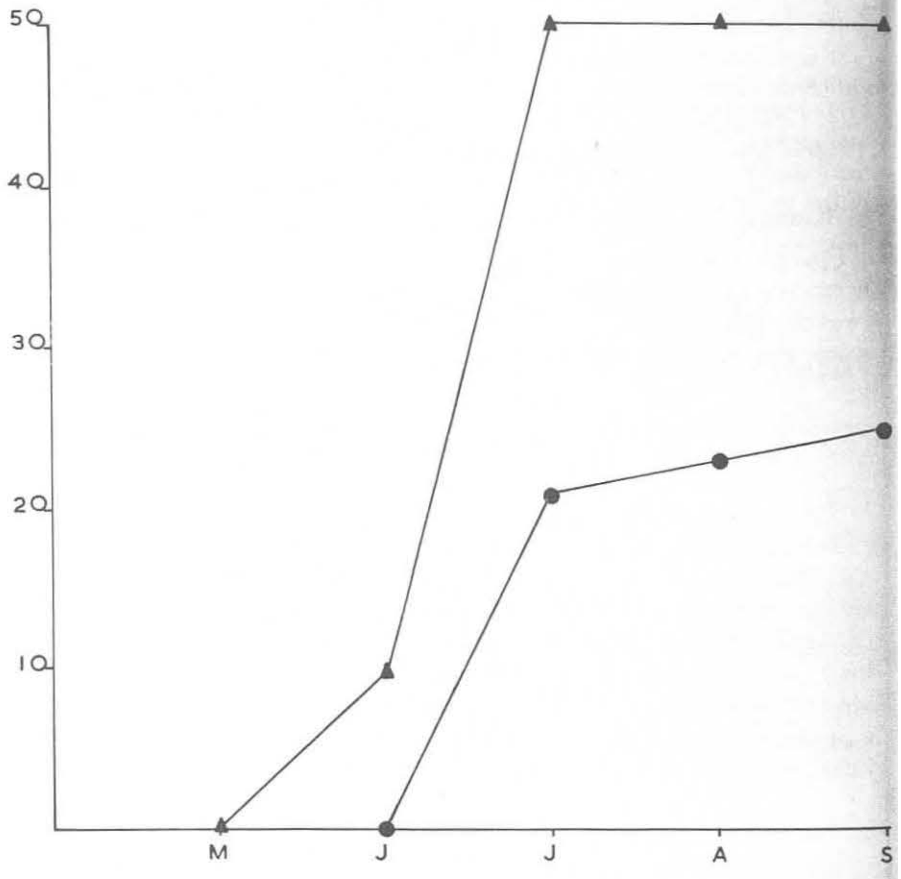

Fig. 1. The successive changes in the average number of *Sporobolomyces* colonies per cm^2 of ten leaf surfaces.

▲ — ▲ upper surface; ● — ● lower surface

Spores of *Cladosporium* were found germinating on the leaf surface in July, when other actively growing colonies were seen, some of which

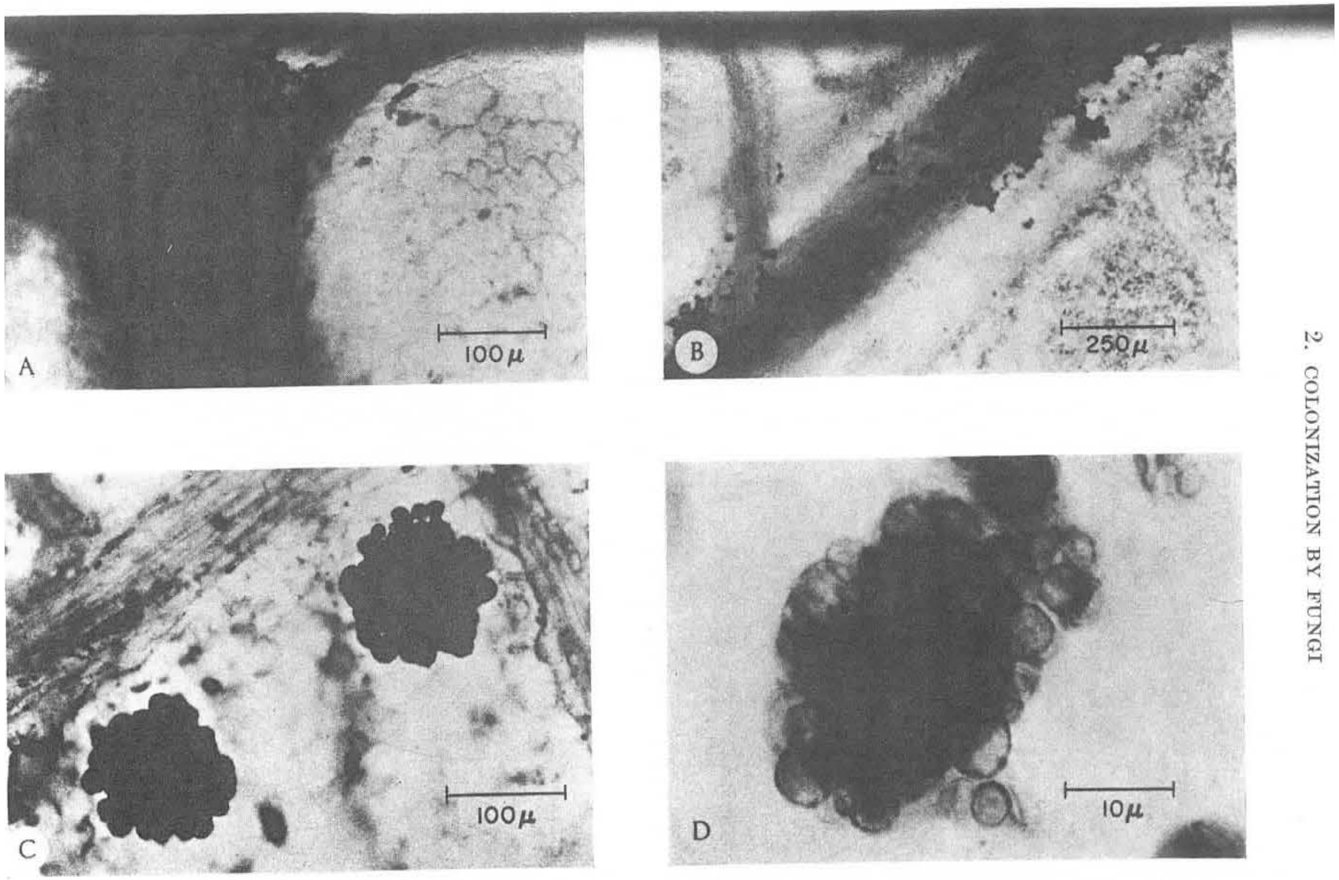

FIG. 2. Cleared leaves of sycamore.

A. Chlamydospores and microsclerotia, mainly of *Aureobasidium*, arranged linearly along the veins (June).
B. "Sooty mould complex" of *Aureobasidium* and *Cladosporium* on the veins (July).
C. Mature sporodochia of *Epicoccum* associated with the veins (November).
D. Young sporodochium of *Epicoccum* with immature conidia (July).

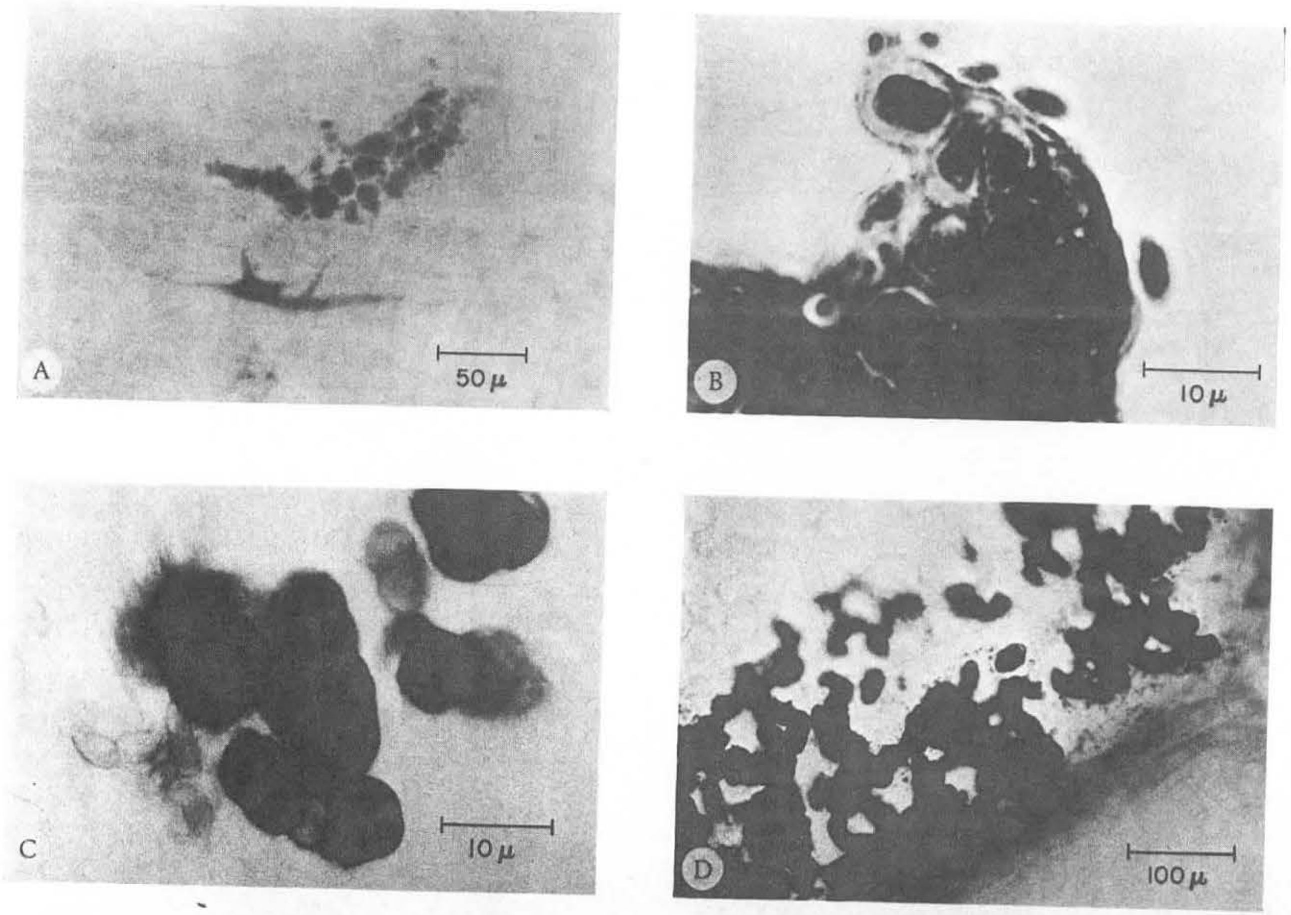

FIG. 3. Cleared leaves of sycamore.

A. Young, developing colony of *Aureobasidium* (above) embedded in mucilage, and a germinating conidium of *Cladosporium* (July).

B. Young colony of *Aureobasidium* showing thickened walls and the beginning of pigmentation (July).

C. Clustered chlamydospores (microsclerotia) of *Aureobasidium* (July).

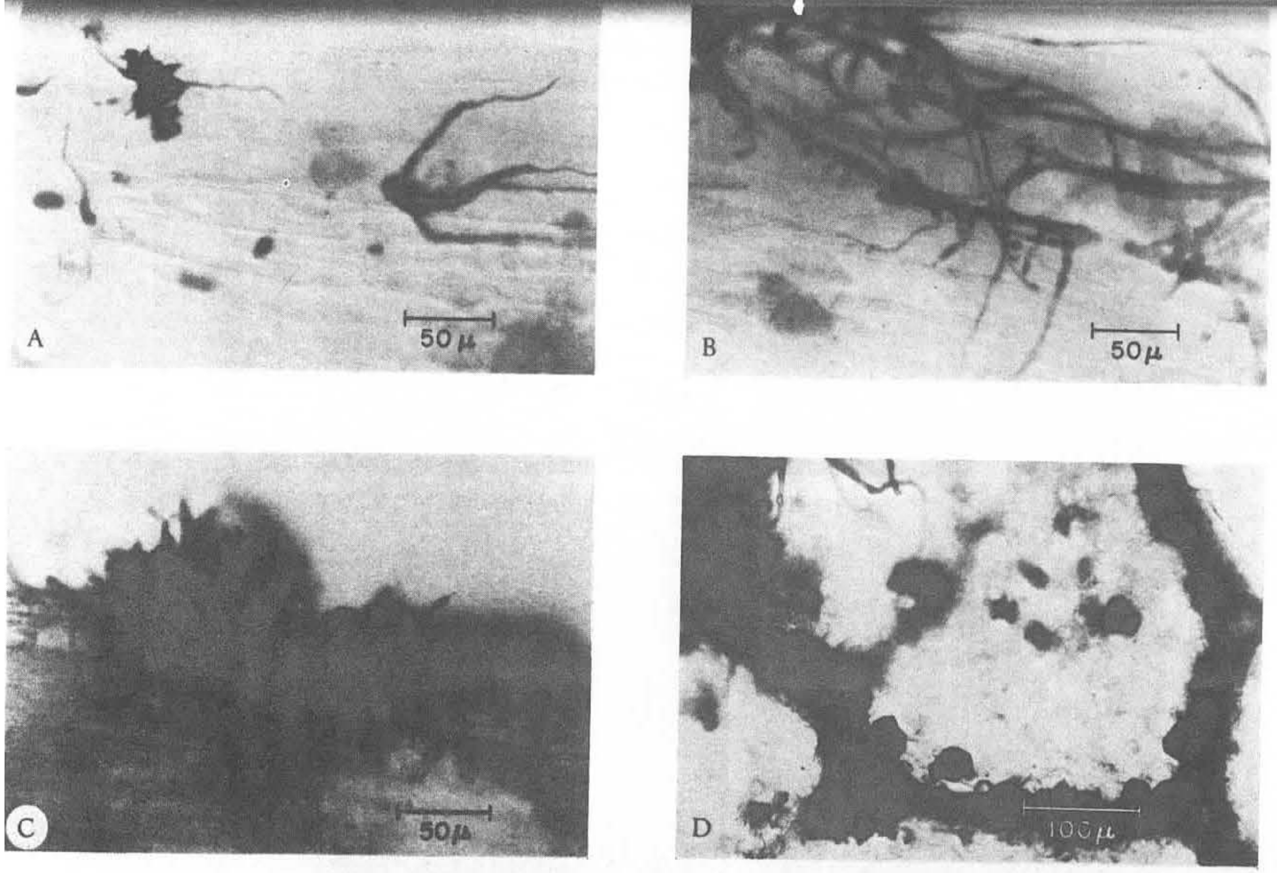

FIG. 4. Cleared leaves of sycamore.

A. Germinating conidia of *Cladosporium* (July).
B. Hyphae of *Cladosporium* covering a vein (July).
C. Hyphae and conidia of *Cladosporium* (July).
D. Microsclerotia of *Cladosporium* associated with veins of a decomposed leaf (February).

were sporulating (Fig. 4 A, B and C). Hyphae of *Cladosporium* were found on the leaf surface in each succeeding month, and these were normally associated with conidiophores. However, by the February following leaf fall, *Cladosporium* appeared to be present only as microsclerotia, which were arranged along the veins (Fig. 4 D). In addition, *Mycosphaerella* spp., some of which may have represented the perfect state of *Cladosporium*, were present on the leaves at this time.

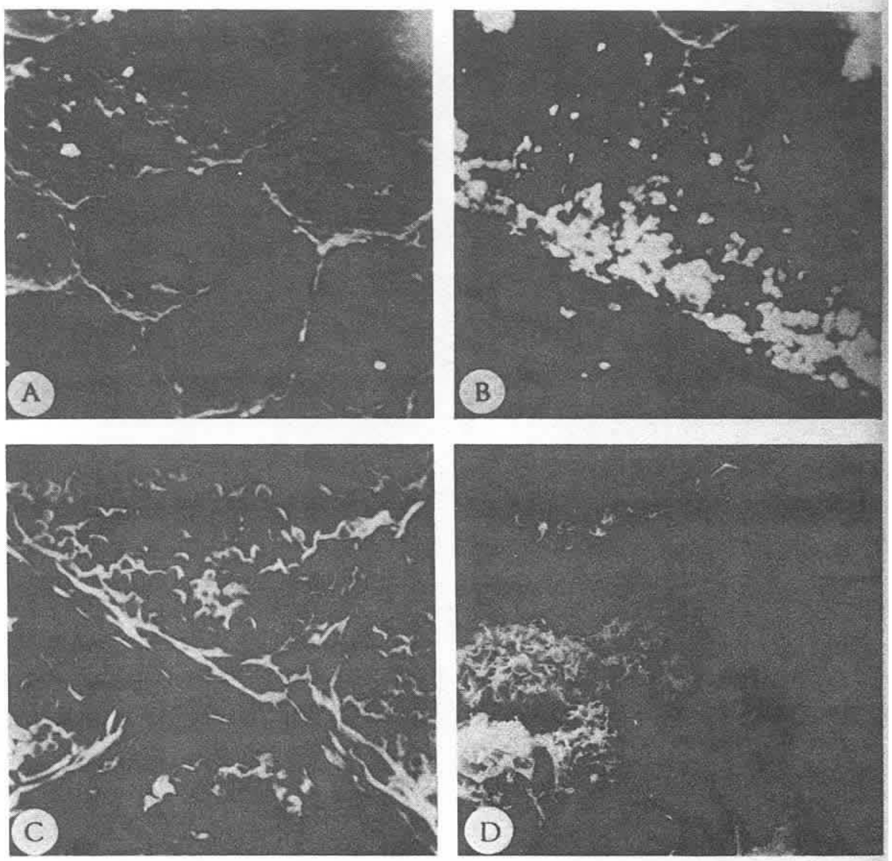

FIG. 5. "Stereoscan" view of sycamore leaf surfaces.

- A, B. Smooth upper surface, with fungal colonies present on a vein in B. (A × 115; B × 57).
- C. Papillate lower surface, with protruding veins (× 125).
- D. Lower surface: Crystalline, waxy excrescences on lower surface (× 6300) and smaller waxy flakes on the veins × 1054.

Young sporodochia of *Epicoccum nigrum* were first seen in July (Fig. 2 D), and were found with increasing frequency until after leaf fall.

In addition to these three important phylloplane fungi, spores of other fungi such as *Alternaria*, as well as pollen grains and algal cells, were recorded from time to time.

3. Scanning Electron Microscopy

The use of the scanning electron microscope confirmed the veinal distribution of fungi on freshly collected leaves which had received no treatment other than coating with gold and desiccation before examination. This instrument also showed differences in the topography of the two leaf surfaces. While the upper surface appeared bare except for the presence of fungal colonies, the lower surface showed stomata, protruding veins, and many crystalline waxy excrescences (Fig. 5). Fungal structures were only rarely seen on the lower surfaces examined.

4. Cultural examination

(*a*) *Washed leaves.* The results obtained from plating out washed leaf squares on P.D.A. are recorded in Fig. 6, where the percentage frequency of isolation refers to the number of times each species was isolated from 50 leaf squares. *Aureobasidium* was found on all leaves from the first opening of the buds, and it remained at this level until after the leaves were shed. *Cladosporium* was infrequently recorded in May, but was present on all leaf squares examined in July. This abundance coincided with the normal summer peak of *Cladosporium* in the atmosphere. *Epicoccum* was recorded in June, and became more frequent from July onwards, attaining its maximum frequency after leaf fall. *Alternaria* spp. and *Botrytis cinerea* were infrequently recorded on the green leaves, and both were sporadic in their occurrence on the two media used.

(*b*) *Surface-sterilized leaves.* The frequency of occurrence of the fungi isolated from surface sterilized leaves is illustrated in Fig. 7. *Aureobasidium* was first isolated from these leaves within three weeks of the opening of the buds. It occurred in all leaves examined, but not in all leaf squares cut from these leaves. On the leaf squares, it was most frequently recorded in June, and thereafter was found in about 20% of the squares which were examined. *Cladosporium* was isolated from a few leaf squares in June, and was present in about 20% of them from September onwards, although by then it was present in all complete leaves. *Epicoccum*, *Alternaria* and *Botrytis* were only recorded from obviously senescing leaves from September onwards, but all increased in abundance after leaf fall.

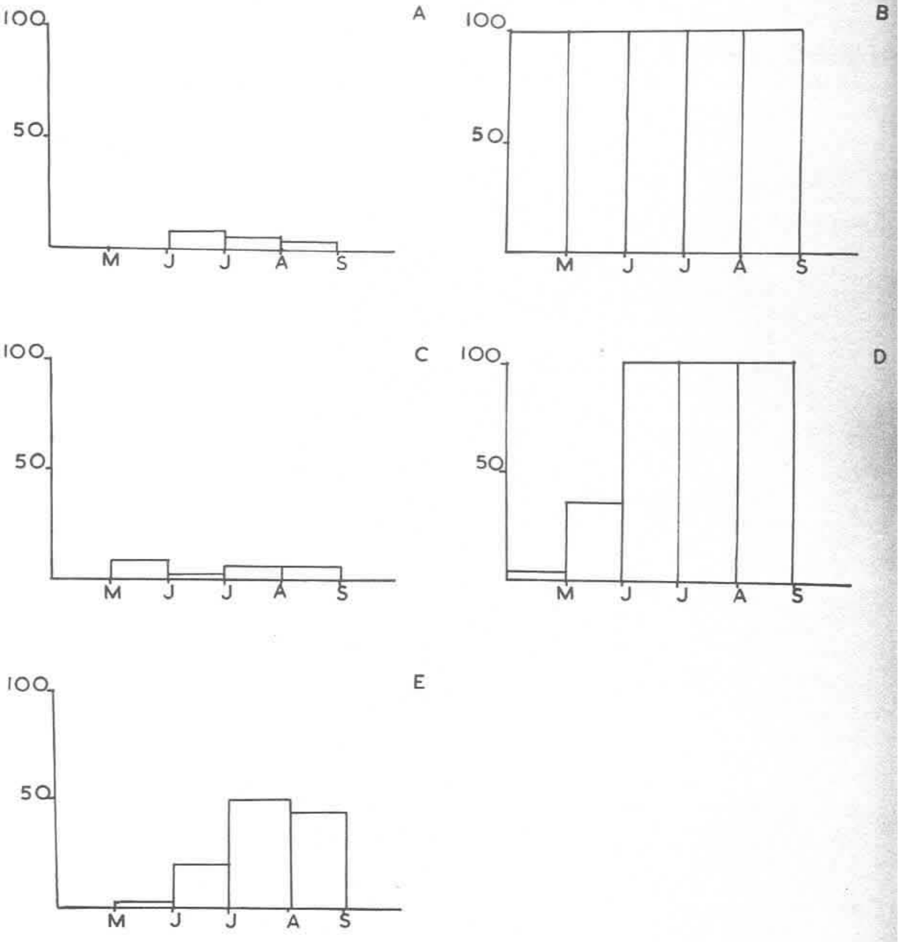

FIG. 6. Percentage frequency of isolation on monthly samples of 50 washed leaf squares.

A. *Alternaria tenuis.*
B. *Aureobasidium pullulans.*
C. *Botrytis cinerea.*
D. *Cladosporium herbarum.*
E. *Epicoccum nigrum.*

5. Leaf digestion

In order to investigate further the presence of the fungi recorded from surface sterilized leaves, the veins remaining after randomly selected leaf squares had been digested with pectinase were plated out. After incubation, these veins yielded *Aureobasidium* in about the same

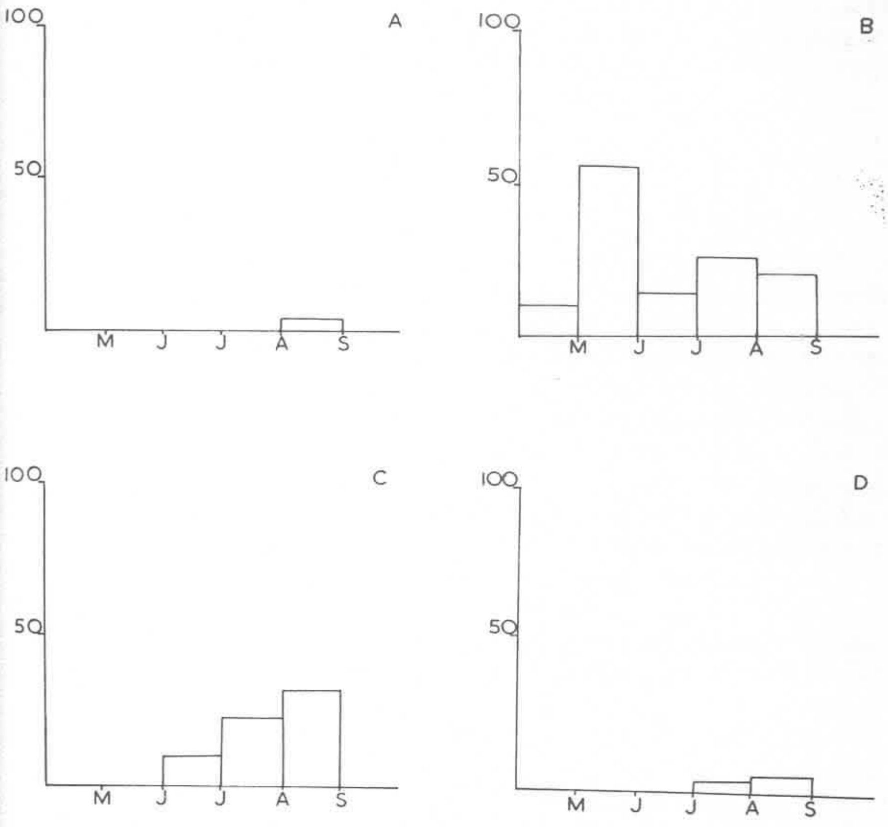

FIG. 7. Percentage frequency of isolation on monthly samples of 50 surface-sterilized leaf squares.
A. *Alternaria tenuis.*
B. *Aureobasidium pullulans.*
C. *Cladosporium herbarum.*
D. *Epicoccum nigrum.*

proportion as it was recorded on the squares taken from the surface sterilized leaves. *Cladosporium*, which was sometimes found in these veins, did not appear to be restricted to them.

6. Exposure to ultra-violet radiations

The survival of *Cephalosporium* sp. and the hyaline conidia of *Aureobasidium* was greatly reduced after 5 min exposure and was nil after 10 min. A majority of the spores of *Cladosporium* were killed after 10 min exposure, but some survived for 35 min. *Alternaria* and *Epicoccum* survived at high levels throughout the experimental time of exposure, as did the dark, thick-walled chlamydospores and microsclerotia of *Aureobasidium*. Individual cells of *Sporobolomyces* were killed after exposure for 5 min, while colonies, both on the leaf surface and on agar plates, yielded living spores after exposure for 35 min.

DISCUSSION

The examination of the surfaces of green sycamore leaves by several different methods has shown the presence of actively growing fungi from the earliest stages of leaf development after the buds opened. The distribution of these fungi, however, was not random. *Sporobolomyces* was found predominantly along the veins on the upper surface, and it was relatively rare on the lower surface. Last (1955) recorded *Sporobolomyces* as occurring equally abundantly on both surfaces of barley leaves where it was present between the veins. On Chrysanthemum, it was present mainly on the lower surface, and on elm it was found along the veins (Last and Deighton, 1965).

The veinal distribution of other fungi, particularly *Aureobasidium* and *Cladosporium*, has also been found in cleared leaves, where different stages of development have been seen. It has been suggested (Dickinson, 1967) that clearing techniques may disturb the spatial arrangements of fungi on leaves. However, the distribution of fungi found on cleared sycamore leaves has been confirmed by the use of a scanning electron microscope when there was no prior treatment of the leaves which could displace fungal structures. Other workers have referred to the veinal distribution of fungi. Pesante (1963) described the localization of *Aureobasidium* on the veins of lime, and suggested that this may be due to the senile condition of the leaf when honeydew appeared, and not to the presence of insects as he could not find any on the leaves. Friend (1965) also referred to the presence of sooty moulds on and near veins on the undersurface of lime leaves. Dickinson (1967), using leaf impression strips, found numerous yeast-like cells above the vascular strands of pea leaves.

There are a number of possible reasons for the veinal distribution of fungi. These include:

(i) spores may be washed into the channels over the veins on the upper leaf surface.
(ii) there may be more exudates along the veins, particularly of senescing leaves, as suggested by Pesante (1963).
(iii) vein extension cells may bring nutrients nearer the surface along the veins (Wylie, 1943), and thus facilitate exudation.

(iv) aphid punctures may release more exudates, and may also provide a means of entry for fungi to penetrate into the leaf.
(v) the fungi may be present inside the leaf, located within the veins, from where it could reach the surface along the veins.

The presence of actively growing fungi on living leaves has been shown, for example, by Dickinson (1965), who differentiated between those species such as *Cladosporium* which grew and sporulated immediately, and others such as *Ascochytula* which grew as sterile mycelia on the living leaf, and produced their fructifications after leaf senescence. Hogg and Hudson (1966) recognized three groups of fungi occurring on beech leaves. Their first group, comprising the primary colonizers, included *Alternaria*, *Aureobasidium*, *Botrytis* and *Cladosporium* which have commonly been found on other plants including sycamore. The present results, however, indicate that this is not a uniform group of fungi which are all actively growing together in this exacting environment, where only a relatively small number of species are able to survive. Amongst them, competition plays a very large part in determining their period of active growth, so that there is a succession even within these primary colonists. *Aureobasidum*, which would appear capable of unrestricted growth, was spatially confined to the surfaces and interior of the veins, where its active growth was confined to the period between the bursting of the bud and the arrival in quantity of *Cladosporium* and *Epicoccum*. On the leaf surface it then became thick-walled and pigmented (Fig. 3 C and D). Similar chlamydospores and microsclerotia form when *Aureobasidium* is grown in paired cultures with *Cladosporium* and particularly with *Epicoccum*. In this condition, *Aureobasidium* is as resistant to ultra-violet light, as are the conidia of *Alternaria* and *Epicoccum*. It also seems probable that the chlamydospores and microsclerotia of *Aureobasidium* are inactive on the leaf surface. These resting structures germinate easily and rapidly on agar, so that this fungus can be isolated from leaves even though it may not then be actively growing on their surfaces. If this is so, then later isolations of *Aureobasidium* from leaf litter and from the soil would appear to be from these inactive structures and not from growing hyphae.

The isolation of *Aureobasidium* from the veins following leaf digestion indicates that it must have grown actively within the leaf, where its period of active growth would be longer than on the leaf surface, as *Cladosporium* only began to penetrate inside the leaf in July, and *Epicoccum* in August.

The appearance of *Cladosporium* on the leaf surface also changed with the passage of time. It first appeared as spores in May and by July there were abundant hyphae with conidiophores (Fig. 4 B and C). However, by the February following leaf fall, it was present predominantly as sclerotia (Fig. 4D). In this form it can readily be isolated on agar, and these structures may be the source of at least some of the isolations of *Cladosporium* from the soil, as well as providing a source of inoculum to colonize leaves later in the year.

Epicoccum has not previously been regarded as an important early leaf colonist. Hering (1965) recorded it on the leaves he studied, but did not include it amongst the species which he thought were leaf-inhabiting. Similarly, it was not listed in the main groups described on beech by Hogg and Hudson (1966). However, Friend (1965) found it on lime leaves collected in October, and in the present study it has been shown to be an active colonizer of green sycamore leaves.

The survival of the primary colonizers after exposure to ultra-violet light shows that they are well adapted for this role. The hyaline conidia of *Aureobasidium*, however, were quickly killed. These conidia, which are so characteristic of *Aureobasidium* in culture, have not been found on cleared leaves. The very early stages of *Aureobasidium* on leaf surfaces are in the form of clusters of hyaline cells embedded in mucilage, and these appear able to survive normal exposure to ultra-violet light. Similarly, colonies of *Sporobolomyces* survive when individual cells do not.

Nicot (1960) pointed out that brown pigmentation, generally thick membranes, a high rate of production of mainly multicellular spores and a tendency for chlamydospores to be present in groups, were all characteristics which can afford protection against desiccation and strong light. Pugh and Williams (1968) suggested that protection against, tolerance of, or perhaps even an ability to utilize light energy would appear to confer some advantage to competitive saprophytes under aerial conditions. Many of these features are to be found in the successful leaf surface colonizers.

Several factors appear to be involved in the more rapid decomposition of sycamore leaves compared with the slower rate of oak (Hering, 1965) and beech (Hogg and Hudson, 1966). The morphology, anatomy and content of the leaves all differ. Handley (1954), for example, showed that there is more than twice as much lignin and humified substances in beech and oak as in sycamore litter. A differential amount of tannin and phenolic substances would also affect the speed of decomposition. Green sycamore leaves certainly produced a more substantial growth of the primary colonizers than has been reported for oak and beech, and there seems to be a correlation between this early appearance of fungi with the abundance of aphids on sycamore leaves. A similar correlation was noted by Friend (1965) on lime leaves.

The subsequent colonization of sycamore leaves showed a much greater degree of overlapping of the secondary and tertiary species than was found on beech. The process of decomposition in sycamore is thus found to be initiated earlier than in the other plants studied, and to be completed within a relatively short period of time.

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